SYSTEM AND METHOD FOR XENOBIOTIC CELL AND TISSUE CULTURE SUPPORTED BY SURROGATE AVIAN VASCULAR CIRCULATION

FIELD OF THE INVENTION

The invention relates to cell, tissue and organ culturing and engineering. In particular, the invention relates to the use of avian embryonic cultures for and with the same.

BACKGROUND OF THE INVENTION

Cells or tissues intended for therapeutic or cosmetic applications are typically cultured using standard *in vitro* culture techniques. However, these techniques suffer from a host of shortcomings; one of the most significant being the inability to foster vascularization (*i.e.*, angiogenesis) in the subject culture. The lack of vascularization presents a substantial obstacle for many tissue engineering applications, as the local architecture of tissues and organs is intimately tied to and relies upon the development of a circulatory vasculature. By way of example, it has been found that insulin-secreting cells generally do not function properly (*i.e.*, they do not secrete insulin) without capillary contact. As is well known in the art, blood vessels are required for, *e.g.*, the local delivery of nutrients and oxygen to cells. Without these vessels, the growth and proliferation of tissue cultures into more sizable cultures, organs and complete tissue structures is limited. Because cells must remain in rather close proximity to a source of nutrients and oxygen to survive, tissue-engineered constructs or cultures generally remain only a few millimeters thick, absent a circulatory vasculature.

There is thus a need in the art for a system and method for culturing cells, organs and tissues that provides a circulatory vasculature. Such a system and method may find application in various aspects of tissue and organ engineering, as well as in studying and supporting cell growth, differentiation and proliferation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a chorioallantoic membrane ("CAM") from an avian egg in accordance with an embodiment of the present invention. The CAM is a highly vascular membrane that forms in the embryo-air interface and conducts oxygen interchange to sustain the avian embryo.

Figure 2 depicts the preparation of *ex ovo* avian cultures in accordance with an embodiment of the present invention. Fig. 2A depicts the opening of an avian egg under sterile conditions. Fig. 2B depicts a series of *ex ovo* avian eggs in cylindrical culture dishes. Fig. 2C depicts the series of *ex ovo* avian eggs once the CAM has developed and migrated toward the upper surface thereof (*i.e.*, the air interface). Fig. 2D depicts the placement of a xenograft upon a CAM.

Figure 3 depicts a CAM from a Japanese quail egg in accordance with an embodiment of the present invention.

Figure 4 depicts a mouse skin xenograft grafted directly onto the CAM in accordance with an embodiment of the present invention. Fig. 4A depicts the xenograft 24 hours following deposit onto the CAM; Fig. 4B depicts the same xenograft 48 hours following deposit onto the CAM.

Figure 5 depicts a mouse skin xenograft transplanted upon a CAM in accordance with an embodiment of the present invention.

Figure 6 depicts a mouse skin xenograft transplanted upon a CAM in accordance with an embodiment of the present invention.

Figure 7 depicts a hair-containing mouse skin xenograft transplanted upon a CAM in accordance with an embodiment of the present invention.

DESCRIPTION OF THE INVENTION

All references cited herein are incorporated by reference as if fully set forth.

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton *et al.*, Dictionary of Microbiology and Molecular Biology 2nd ed., J. Wiley & Sons (New York, NY 1994); March, Advanced Organic Chemistry Reactions, Mechanisms and Structure 4th ed., J. Wiley & Sons (New York, NY 1992); and Sambrook and Russel, Molecular Cloning: A Laboratory Manual 3rd ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY 2001) provide

one skilled in the art with a general guide to many of the terms used in the present application. One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

"Gene transfer" or "gene delivery" refers to methods or systems for reliably inserting foreign DNA into host cells. Such methods can result in transient expression of non-integrated transferred DNA, extrachromosomal replication and expression of transferred replicons (*e.g.*, episomes), or integration of transferred genetic material into the genomic DNA of host cells. A number of systems have been developed for gene transfer into mammalian cells. *See, e.g.,* U.S. Patent No. 5,399,346.

The term "transfection" is used herein to refer to the uptake of foreign DNA by a cell. A cell has been "transfected" when exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. See, *e.g.*, Graham *et al.* (1973) Virology, 52:456, Sambrook and Russel, Molecular Cloning: A Laboratory Manual 3rd ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY 2001), Davis *et al.* (1986) Basic Methods in Molecular Biology, Elsevier, and Chu *et al.* (1981) Gene 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties, such as a plasmid vector and other nucleic acid molecules, into suitable host cells. The term refers to both stable and transient uptake of the genetic material.

"DNA" is meant to refer to a polymeric form of deoxyribonucleotides (*i.e.*, adenine, guanine, thymine and cytosine) in double-stranded or single-stranded form, either relaxed or supercoiled. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes single- and double-stranded DNA found, *inter alia*, in linear DNA molecules (*e.g.*, restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (*i.e.*, the strand having the sequence homologous to the mRNA). The term captures molecules that include the four bases adenine, guanine, thymine and cytosine, as well as molecules that include

base analogues which are known in the art.

A "gene" or "coding sequence" or a sequence which "encodes" a particular protein is a nucleic acid molecule that is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences; although one of skill in the art will readily appreciate that various polynucleotides do not operate in this fashion (*e.g.*, antisense RNA, siRNA, ribozymes, wherein the RNA transcript is the product). With respect to protein products (*i.e.*, not RNA products), the boundaries of the coding sequence are determined by a start codon at the 5' (*i.e.*, amino) terminus and a translation stop codon at the 3' (*i.e.*, carboxy) terminus. A gene can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the gene sequence. Moreover, a "gene" (i) starts with a promoter region containing multiple regulatory elements, possibly including enhancers, for directing transcription of the coding region sequences; (ii) includes coding sequences, which start at the transcriptional start site that is located upstream of the translational start site and ends at the transcriptional stop site, which may be quite a bit downstream of the stop codon (a polyadenylation signal is usually associated with the transcriptional stop site and is located upstream of the transcriptional stop); and (iii) may contain introns and other regulatory sequences to modulate expression and improve stability of the RNA transcript.

The term "control elements" refers collectively to promoter regions, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for the replication, transcription and translation of a coding sequence in a recipient cell. Not all of these control elements need always be present, so long as the selected coding sequence is capable of being replicated, transcribed and translated in an appropriate host cell.

The term "promoter region" is used herein in its ordinary sense to refer to a nucleotide region including a DNA regulatory sequence, wherein the regulatory sequence is derived from a gene which is capable of binding RNA polymerase and initiating transcription of a downstream (3'-direction) coding sequence.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control elements operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control elements need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

For the purpose of describing the relative position of nucleotide sequences in a particular nucleic acid molecule throughout the instant application, such as when a particular nucleotide sequence is described as being situated "upstream," "downstream," "5'," or "3'" relative to another sequence, it is to be understood that it is the position of the sequences in the non-transcribed strand of a DNA molecule that is being referred to as is conventional in the art.

"Isolated" as used herein when referring to a nucleotide sequence, refers to the fact that the indicated molecule is present in the substantial absence of other biological macromolecules of the same type. Thus, an "isolated nucleic acid molecule which encodes a particular polypeptide" refers to a nucleic acid molecule that is substantially free of other nucleic acid molecules that do not encode the subject polypeptide. However, the molecule may include some additional bases or moieties that do not deleteriously affect the basic characteristics of the composition.

"Mammal" as used herein refers to any member of the class Mammalia, including, without limitation, humans and nonhuman primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs, and the like. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be included within the scope of this term.

The invention is based on the inventor's use of *ex ovo* avian cultures to sustain *in vitro* proliferation, expansion and differentiation of specific cell types, tissues and organs. The system and method of the invention can be used to grow, expand and/or differentiate cells, tissues or organs that require or can benefit from vascularization;

although a need for or the capacity to benefit from vascularization is not a requirement for any particular cells, tissues or organs to be suitable for use in connection with the present invention, as will be readily appreciated by one of skill in the art. However, the invention does have the distinct advantage of providing cultured biological materials with a rich vascular supply. As noted above, this is known to be important in maintaining viability by providing cells with nutrients and oxygen. Moreover, the embryonic blood supply is abundant in growth factors and hormones that generally promote cell growth and differentiation. Furthermore, tissue explants, live cells or organs from any species may be successfully cultured with the *ex ovo* system and methods of the instant invention, because an immunologic response does not occur in the embryonic culture environment. In fact, the avian embryo and its extraembryonic membranes are immune-incompetent. There is thus no concern of rejection in the culture/growth environment.

In one embodiment of the present invention, a fertilized avian egg may be used. The avian egg may be from any suitable species of bird, as will be readily appreciated by one of skill in the art. By way of example, a Japanese quail (Cortunix cortunix japonica) egg may be utilized in connection with various embodiments of the present invention. Eggs from still further, non-avian organisms may be used in alternate embodiments of the invention.

As described in greater detail in the ensuing discussion, in various embodiments of the present invention, a wide array of cells, tissues and organs may be grown and/or cultured. While the cells, tissues and organs may be harvested or derived from the same species (indeed, even from the very same organism) from which the egg used in the *ex ovo* system is obtained, in most embodiments of the invention, the cells, tissues and organs are xenografts (*i.e.*, they are harvested, derived or otherwise obtained from a species other than that from which the egg is obtained). In these latter embodiments of the instant invention, cells, tissues or organs from one species are grown and/or cultured on an *ex ovo* egg of another species. This may be beneficial in, for instance, providing a vasculature to support the growth and/or culture of the xenograft. However, because the vasculature for the xenograft is at least partially developed from the *ex ovo* avian egg, avian cells may be found in the xenograft vasculature (*e.g.*, avian endothelial cells in the xenograft vasculature) and/or may appear elsewhere (*i.e.*, by migration or otherwise) in the xenograft itself.

In light of this feature of the present invention, it may be convenient to select an avian egg for use with the inventive system and methods for which tools are readily (*e.g.*, commercially) available for the screening and/or removal of avian cells in/from the xenograft. As such, the Japanese quail egg is but one example of a convenient egg for use in connection with various embodiments of the instant invention, at least in part, because antibodies are readily available that may be used to identify Japanese quail egg cells present in the xenograft or its vasculature. Once so identified, various methodologies may be employed to account for these cells in the further analysis of the xenograft; to remove these cells either prior to or following transplantation of the xenograft to a recipient; or to address the immunological response that a transplant recipient may exhibit upon transplantation. For example, in one embodiment of the instant invention, a section of mammalian skin (*e.g.*, human skin) is grown on a Japanese quail egg and thereafter transplanted to a mammal (*e.g.*, a human). The transplant recipient's immune system may be artificially suppressed by routine techniques to address potential tissue rejection based on the presence of the avian cells in the skin graft.

In an alternate embodiment of the present invention, the animal from which the egg is obtained may be transgenic. A bird, such as a chicken or any other egg-laying animal in which a transgene may be readily introduced, may be genetically engineered to carry an inducible gene that causes apoptosis (*i.e.*, programmed cell death) in its cells. By way of example, apoptotic genes that may be used in accordance with this embodiment of the invention may include, but are in no way limited to, genes of the Myc family, and genes encoding agnoproteins (*e.g.*, agnoprotein 1a, agnoprotein 1b), which are small proteins of the primate polyomaviruses simian virus 40 (SV40), BK virus (BKV) and JC virus (JCV) that are encoded by an open reading frame (ORF) located near the 5'-end of the late bicistronic mRNA that also encodes the structural protein VP1, as described in R. Johne et al., *"Agnoprotein 1a and agnoprotein 1b of avian polyomavirus are apoptotic inducers,"* Journal of General Virology, 81:1183-1190 (2000), the disclosure of which is incorporated by reference herein in its entirety. Moreover, the preparation of transgenic chickens, which may be used in accordance with one embodiment of the present invention, is described in P.E. Mozdziak *et al.*, *Development of Transgenic Chickens Expressing Bacterial β-Galactosidase,*

Developmental Dynamics, 226:439-445 (2003), the disclosure of which is incorporated by reference herein in its entirety.

When an egg from the transgenic animal is used in connection with the *ex ovo* system and methods of the instant invention, and cells derived therefrom appear in the xenograft vasculature and/or elsewhere in the xenograft itself, the transgene can be induced by routine methods; for instance, by triggering the promoter of the transgene, or by triggering another control element that is operably linked to the transgene to regulate its transcription. Regardless of the manner in which transcription of the transgene is induced, its induction results in apoptosis of the cells from the transgenic animal, thereby eliminating them from the xenograft. The transgene can be induced either prior to or following transplantation of the xenograft to a recipient. In fact, this may entirely eliminate the need for artificial immune suppression in the recipient; although, in an alternate embodiment of the present invention, artificial immune suppression may be implemented in combination with the induction of an appropriate transgene.

In practice, in one aspect of the invention, a fertile egg is incubated for an initial period *in ovo*. The duration of this initial period may vary based upon the species of egg used, but, for avian eggs, the initial period may generally be from about three to about five days. During this initial period, the egg may be incubated in a commercial egg incubator at conditions generally conducive to support incubation (*i.e.*, accounting for temperature, humidity and other factors that will be readily appreciated by those of skill in the art).

Following the initial period of *in ovo* incubation, the egg may be opened under sterile conditions (Fig. 2). In one embodiment of the present invention, the egg is opened through its air chamber and the exterior shell of the egg is removed, either in whole or in part. Once the egg is opened, its contents may be placed in a culture dish. In one embodiment of the present invention, the culture dish is plastic. Furthermore, in an embodiment of the present invention, a generally cylindrical culture dish slightly larger than the overall diameter of the egg yolk may be used. In an alternate embodiment, the internal contents of multiple eggs may be placed in the same culture dish. This may provide a more expansive surface area for cell, tissue and organ culture. Moreover, variant sizes and configurations of culture dishes may be used,

depending upon the size and shape of the egg or eggs, the orientation of the internal components and anatomy of the egg or eggs, and other factors relating to the convenience of manipulating the egg and the cell culture placed therein or thereupon. While not wishing to be bound by any particular theory, it has been demonstrated and it is believed that, as it develops, the chorioallantoic membrane ("CAM") of the egg orients itself to face the air interface of the culture dish with the local atmosphere (Fig. 3).

Once the egg (or eggs) is opened and placed in a suitable culture dish, it may be cultured for an additional period *ex ovo*, until a well-vascularized CAM covers the surface of the culture dish (Figs. 2 and 3). In the alternate embodiment of the present invention discussed above that includes multiple eggs, the CAMs of the eggs may fuse or bind together to create a unitary CAM across multiple eggs (not shown). Furthermore, as with the initial period described above, the duration of the additional period may vary based upon the species of egg used, but, for avian eggs, the additional period may generally be from about 24 to about 48 hours. During this additional period, the egg may be maintained at an ambient temperature of about 37°C.

Following the additional period of *ex ovo* culture, a biological material may be deposited upon the surface of the CAM. This biological material may be selected from any biological material suitable for culture, growth, differentiation or any combination thereof in the system of the invention. Suitable biological materials may include, but are in no way limited to, organs or fragments of organs, tissues or tissue sections, cell suspensions or physical three-dimensional matrices containing embedded live cells. The biological material may thereafter remain viable and capable of further expansion and/or differentiation. Furthermore, tissue explants or live cells from any species may be successfully cultured in this fashion, because an immunologic response does not occur in the embryonic culture environment. In fact, the avian embryo and its extraembryonic membranes are immune-incompetent. Thus, cells, tissues or organs may be xenotransplanted without risk of rejection. By way of example, Figs. 4, 5, 6 and 7 depict mouse skin xenografts that were transplanted onto the CAMs from Japanese quail eggs. In particular, as depicted in Fig. 4, the transplanted xenograft recruits vessels from the CAM, and the xenograft is kept alive by "borrowing" from the CAM circulation. In an alternate embodiment of the present

invention, biological material may be xenotransplanted underneath the CAM within the embryo proper or in the amniotic chamber.

Once deposited upon or xenotransplanted underneath the CAM, the biological material may be cultured for a culture period to provide sufficient time for embryonic vessels to develop into it. In avian systems, this culture period may be from about eight to about fifteen days. During this culture period, the embryonic vessels develop into the biological material and thereby provide the deposited/implanted biological material with a permeable vascular network that transports oxygen and other nutrients necessary to ensure viability and promote proliferation and differentiation. Growth factors, hormones and other compounds may be transported by way of this vascular network, as well.

In an alternate embodiment of the present invention, a scaffold support structure may be included on the surface of the CAM to support the organization and/or anchoring of xenografts thereto. In one aspect of the invention, this scaffold support structure may include a piece of eggshell from the egg that is left upon the CAM following removal of the egg contents from the eggshell. In other embodiments, this scaffold support structure may be selected from other biological or non-biological supports.

Furthermore, xenografts grown and/or cultured in connection with the *ex ovo* system and methods of the present invention may be moved from one egg (or collection of eggs) to another egg (or collection of eggs) to support continual growth and/or culture. This may be necessary if, for example, it is desirable for a xenograft to be supported for a longer period of time than the *ex ovo* egg can survive.

While the description above refers to particular embodiments of the present invention, it will be understood that many modifications may be made without departing from the spirit thereof. The accompanying claims are intended to cover such modifications as would fall within the true scope and spirit of the present invention. The presently disclosed embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims, rather than the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.